NOVEL ANTI-VIRAL VSF PROTEIN AND HYBRIDOMA PRODUCING THE SAME

TECHNICAL FIELD

The present invention relates, in general, to a novel viral-inhibitory substance, and more particularly to a virus suppressing factor (VSF) having antiviral activity, which is produced in an immune cell infected with a variant of the encephalomyocarditis virus (EMCV), EMC-DV. In detail, the present invention relates to a VSF protein having inhibitory activity against a variety of viruses, produced in an immune cell infected with a variant of the encephalomyocarditis virus, EMC-DV, a method of preparing such a VSF protein, a hybridoma secreting such a VSF protein, and a method of preparing such a hybridoma. In addition, the present invention is concerned with a pharmaceutical composition comprising the VSF protein for prevention or treatment of viral infections, and a method of preventing or treating viral infections using such a pharmaceutical composition.

PRIOR ART

The conventional antiviral agents are largely classified into synthetic chemical compounds and bio-derived molecules. The synthetic chemical compounds, which were mainly developed to act only against specific viral diseases, are disadvantageous in that they have several side effects, and viruses easily develop resistance thereto.

Cytokines such as interferons (IFNs) are well known as bio-derived anti-viral agents. The cytokines differ from the synthetic compounds in terms of not having severe side effects. However, there are disadvantages to cytokines, as follows: they have limitations in their practical use and low efficacy in vivo.

On the other hand, one subject infected with HIV has been found not to develop AIDS,

suggesting that his immune system has a potential mechanism to inhibit HIV function. Recently, it has been reported by scientists that virus-infected $CD8^+$ cells function to inhibit some viral functions by secreting a new soluble molecule.

After in vitro culturing $CD8^+$ cells derived from HIV-infected individuals not developing AIDS, when HIV-infected cells were treated with the culture supernatant of the $CD8^+$ cells, it was found that HIV proliferation is suppressed, and that the $CD8^+$ cells secreted a soluble antiviral substance (Walker CM et al., Science, 234, 1563-1566(1986); and Walker CM et al., Immunology, 66, 628-630 (1989)). Such a soluble antiviral substance was also discovered in SIV-infected monkeys and FIV-infected cats. In addition, it has been reported that HIV proliferation is suppressed by a soluble antiviral substance secreted by $CD8^+$ cells infected with herpes virus. This finding was very interesting in light of the fact that an antiviral substance secreted by immune cells infected with a specific virus is effective in inhibiting functions of a different virus.

Such an antiviral substance produced by $CD8^+$ cells is called '$CD8^+$ cell antiviral factor (CAF), and efforts to identify the antiviral factor and its action mechanism has been actively undertaken. CAF seems to affect target cells to induce an antiviral state, rather than to directly act on viruses. Therefore, in contrast to the conventional antiviral agents, CAF is predicted to rarely accompany unexpected side effects or generate CAF-resistant viruses.

US Pat. No. 5,707,814 discloses a $CD8^+$ cell antiviral factor (CAF) having inhibitory activity against HIV, SIV and retrovirus infection, which is isolated from $CD8^+$ cell culture fluids. In International Pat. Publication No. WO99/57272, CAF is disclosed as having a molecular weight of about 8 kDa. Also, CAF isolated from eggs derived from a immunized avian is disclosed in International Pat. Publication NO. WO01/07472A1.

However, in the cases of the aforementioned disclosures, it is hard to produce in vitro $CD8^+$ cells secreting the antiviral substance CAF because $CD8^+$ cells cannot be immortalized, resulting in that CAF is secreted transiently and at a small amount in vitro. For these reasons, in vivo experiments using animals cannot be performed. Moreover, when culture fluid of cells

secreting CAF is diluted up to over 10 times, the antiviral effect of CAF disappears even in vitro.

To overcome the problems encountered in the prior art, the present inventors conducted intensive and thorough research into an antiviral factor. The research, in which a cell line continuously secreting an antiviral substance was established by isolating immune cells stimulated by injection of a variant of encephalomyocarditis virus, and fusing the immune cells with myeloma cells to produce hybridomas, resulted in the finding that a VSF protein, secreted by the cell line, has strong antiviral activity against a variety of viruses. Based on the finding, the present inventors identified characteristics of the VSF protein.

DISCLOSURE OF THE INVENTION

The present invention provides a virus suppressing factor (VSF) protein having the following properties: (a) it is increasingly produced in an immune cell stimulated by a variant of encephalomyocarditis virus, EMC-DV, (b) it has antiviral activity which is unchanged by immunoprecipitation and immunoneutralization, (c) it is inactivated by proteinase K, (d) it is not one of the group of antiviral cytokines consisting of IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, G-CSF, GM-CSF, TNF-α, TNF-β, IFN-α, IFN-β, IFN-γ, TGF-β, RANTES, MIP-1α, MIP-1β, MIP-1γ, MCP-1, MCP-3, IP-10 and lymphotactin, (e) it comprises about 55 kDa polypeptide (H), about 30 kDa polypeptides (L1 and L2) and about 25 kDa polypeptide (L3), and (f) it has a molecular weight of over about 100 kDa.

In addition, the present invention provides a VSF protein having the following properties: (a) it is increasingly produced in an immune cell stimulated by a variant of encephalomyocarditis virus, EMC-DV, (b) it has an antiviral activity which is unchanged by immunoprecipitation and immunoneutralization, (c) it is inactivated by proteinase K, (d) it is not one of the group of antiviral cytokines consisting of IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-

13, IL-14, IL-15, IL-16, IL-17, IL-18, G-CSF, GM-CSF, TNF-α, TNF-β, IFN-α, IFN-β, IFN-γ, TGF-β, RANTES, MIP-1α, MIP-1β, MIP-1γ, MCP-1, MCP-3, IP-10 and lymphotactin, (e) it comprises about 55kDa polypeptide (H), about 30 kDa polypeptides (L1 and L2) and about 25 kDa polypeptide (L3), (f) it has a molecular weight of over about 100 kDa, (g) the H polypeptide has a DNA sequence designated as SEQ ID NO: 1 and an amino acid sequence designated as SEQ ID NO: 2, and (h) the L3 polypeptide has a DNA sequence designated as SEQ ID NO: 3 and an amino acid sequence designated as SEQ ID NO: 4.

Preferably, the VSF protein of the present invention has antiviral activity to suppress proliferation or replication of a virus belonging to the genus *Orthomyxoviridae*, *Picornaviridae*, *Retroviridae* or *Herpes*.

The present invention provides a method of producing a hybridoma, comprising fusing an immune cell stimulated by a variant of encephalomyocarditis virus, EMC-DV, with a tumor cell, and producing a hybridoma secreting a VSF protein.

The present invention provides a method of preparing a VSF protein, comprising producing a hybridoma secreting a VSF protein by fusing an immune cell stimulated by a variant of encephalomyocarditis virus, EMC-DV, with a tumor cell, culturing the hybridoma, and isolating a VSF protein from a culture fluid of the hybridoma.

In addition, the present invention provides a method of preparing a VSF protein, comprising producing a hybridoma secreting a VSF protein by fusing an immune cell stimulated by a variant of encephalomyocarditis virus, EMC-DV, with a tumor cell, injecting the hybridoma into an animal, and isolating a VSF protein from an ascitic fluid obtained from the animal.

Preferably, the VSF protein of the present invention is isolated from the culture fluid or ascitic fluid using a Blue Sepharose column, a Protein A agarose column, a hydroxyapatite resin column, a FPLC column, or sucrose gradient.

Further, the present invention provides a hybridoma producing a VSF protein, which is prepared by fusing an immune cell stimulated by a variant of encephalomyocarditis virus, EMC-

DV, with a tumor cell.

Preferably, the hybridoma is a hybridoma 4D1B (accession number KCLRF-BP-00052).

Still further, the present invention provides a pharmaceutical composition for prevention and treatment of viral infections, comprising a therapeutically or preventively effective amount of a VSF protein and a pharmaceutically acceptable carrier.

Still further, the present invention provides a method of preventing and treating viral infections, comprising administering a therapeutically or preventively effective amount of a VSF protein to a subject suffering from a viral infection.

BRIEF DESCRIPTION OF THE DRAWINGS

The above and other objects, features and other advantages of the present invention will be more clearly understood from the following detailed description taken in conjunction with the accompanying drawings, in which:

FIG. 1 is a photograph showing a result of SDS-PAGE of a VSF protein according to the present invention (lane 1: VSF purified using a hydroxyapatite resin column; lane 2: VSF purified using a Protein A agarose column; and lane 3: VSF purified using a Blue Sepharose column);

FIG. 2 is a photograph showing a result of fractionation of a VSF protein according to the present invention using Centricon membranes with various pore sizes (A: 50-100 kDa molecular weight; B: 100-500 kDa molecular weight; and C: > 500 kDa molecular weight);

FIG. 3 is a graph showing antiviral activity of ascitic fluids, obtained from mice intraperitoneally administered with a hybridoma according to the present invention, against Encephalomyocarditis virus (EMCV) and Mengo virus, where antiviral activity is analyzed by MTS assay;

FIG. 4 is a graph showing antiviral activity of culture fluid of a hybridoma according to the present invention, against influenza virus;

FIG. 5 is a graph showing antiviral activity of ascitic fluids, obtained from mice intraperitoneally administered with a hybridoma according to the present invention, against influenza virus;

FIG. 6 is a graph showing antiviral activity of ascitic fluids, obtained from mice intraperitoneally administered with a hybridoma according to the present invention, against HIV-1;

FIG. 7 is a graph showing inhibitory effect of culture fluid of a hybridoma according to the present invention on induction of diabetes mellitus by EMCV, where body weight of mice infected with EMCV (group 2) is compared with that of EMCV-infected mice treated with the culture fluid (groups 3 to 5) 1 week after viral infection ;

FIG. 8 is a photograph showing a result of SDS-PAGE of a VSF fraction, which is obtained by FPLC in which a VSF protein according to the present invention is identified to have a molecular weight of about 163 kDa (lanes 3 and 4: about 163 kDa fraction having antiviral activity);

FIG. 9 shows a result of SDS-PAGE of fractions obtained by sucrose gradient centrifugation to identify subunits of a VSF protein according to the present invention (lane 4: fraction obtained in 17.11% sucrose);

FIG. 10 is a photograph showing results of SDS-PAGE and Western blotting after treatment of a VSF protein according to the present invention with endoglycosidase to investigate glycosylation of the VSF protein (lane 1: purified VSF not treated with endoglycosidase; and lane 2: purified VSF treated with endoglycosidase);

FIGS. 11A to 11D are photographs showing results of SDS-PAGE and Western blotting after treatment of a VSF protein according to the present invention with trypsin, cathepsin, papain and pepsin;

FIGS. 12A to 12C are graphs showing heat stability of a VSF protein according to the present invention; and

FIG. 13 is a graph showing a result of ELISA, in which reactivity of a VSF protein

according to the present invention to anti-mouse IgG(H+L) is compared to that of mouse IgG.

BEST MODES FOR CARRYING OUT THE INVENTION

In an aspect of the present invention, there is provided a virus suppressing factor (VSF) protein having antiviral activity against a variety of viruses.

Encephalomyocarditis virus (EMCV) belongs to the genus *Picornaviridae* (Kim YW et al. The Journal of the Korean Society for Microbiology, 30, 671-681 (1995); and Craighead JE, Progress in Medical Virology, 19, 161-214 (1975)). EMCV has various variants including the M, D, B and DV variants.

The M variant of EMCV (EMC-M variant) induces diabetes mellitus in mice at an irregular frequency, and diabetes mellitus is developed in mice infected with the D variant of EMCV (EMC-D variant) at over 90% frequency (Craighead JE and McLane MF, Science, 162, 913-914 (1968)). The B variant of EMCV (EMC-B variant) is known not to cause diabetes mellitus in mice, and can be isolated by plaque purification (Yoon JW et al., J. Gen. Virol, 69, 1085-1090 (1988)). Although the EMC-D and EMC-B variants are almost indistinguishable biochemically and immunologically, the EMC-D variant rarely induces production of IFNs, whereas the EMC-B variant induces high production of IFNs (Yoon JW et al., J. Infect Dis, 147, 155-159 (1983)). In this regard, it seems likely that diabetes mellitus is developed by production of IFNs. On the other hand, it was reported by the present inventors that the DV variant of EMCV (EMC-DV variant) rarely induces production of IFNs as well as diabetes mellitus (Kim YW et al. The Journal of the Korean Society for Microbiology, 30, 671-681 (1995)).

Both of the EMC-D and EMC-DV variants can attach to pancreatic beta cells, and replicate and proliferate in the beta cells in vitro, finally destroying the beta cells. However, in case of in vivo infection, only the EMC-D variant is diabetogenic by infecting and destroying pancreatic beta cells, whereas, in case of the EMC-DV variant, viral replication is stopped 48 hrs

after infection, and viral proteins gradually disappear. The EMC-DV variant initiates replication after infection, but the viral replication is stopped early, not destroying infected cells, resulting in no infiltration of immune cells into islets of pancreas.

The VSF protein of the present invention is a protein of which production is increased in a cell stimulated by the EMC-DV variant. The term "cell", used in the description of the cell stimulated by an EMC-DV variant, refers to an animal cell, and particularly, to an immune cell functioning to protect an individual from viral infection, reject cells derived from other individuals, and remove transformed cells and aged tissues. Preferably, the cell means an immune cell present in the spleen. Examples of the immune cell may include macrophages, granulocytes, T lymphocytes, B lymphocytes, NK cells and LAK cells. The "animal", used in the expression of the animal cell, includes livestock, which are exemplified by cattle, sheep, pigs, horses, dogs, fowls, ducks and turkeys, mammals, which are exemplified by mice, rats, hamsters and humans, as well as fishes, amphibians, reptiles, and birds, and wherein the VSF protein of the present invention displays antiviral activity in the aforementioned animals.

The term "antiviral activity", which the VSF protein of the present invention displays, refers to activity to supress proliferation or replication of a pathogenic virus, and thus reduce, inhibit or prevent a viral infection. The term "pathogenic virus", of which proliferation or replication is inhibited by the antiviral activity, refers to a virus causing a disease in an animal or a human. Examples of the pathogenic virus may include viruses belonging to the genus *Orthomyxoviridae*, *Picorna viridae*, *Retroviridae* and *Herpes*. Representative examples of the pathogenic virus may include EMC virus (EMCV), Mengo virus, influenza virus, HIV, and human cytomegalovirus (HCMV). In particular, the VSF protein of the present invention exhibits antiviral activity against Mengo virus belonging to the genus *Picornaviridae*, as well as influenza virus belonging to the genus *Orthomyxoviridae*, which has a genetic structure and life cycle completely different from EMC virus belonging to the genus *Picorna viridae* (Examples 10 to 11). In addition, the VSF protein effectively inhibits proliferation of HIV-1 belonging to the genus

Retroviridae (Example 12), and has inhibitory effect on proliferation of vesicular stomatitis virus (VSV) (Example 23).

The antiviral VSF protein according to the present invention has the following properties.

1. The VSF protein of the present invention has physiological activity similar to that of cytokines, but has biological activity distinct from that of conventional antiviral cytokines. In particular, the VSF protein is a different kind of protein from the conventionally known antiviral cytokines including IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, G-CSF, GM-CSF, TNF-α, TNF-β, IFN-α, IFN-β, IFN-γ, TGF-β, RANTES, MIP-1α, MIP-1β, MIP-1γ, MCP-1, MCP-3, IP-10 and lymphotactin.

The present inventors performed RT-PCR to investigate expression of specific cytokines in a hybridoma secreting a VSF protein. As a result, the hybridoma according to the present invention was demonstrated not to express specifically any of the aforementioned cytokines (Example 4).

2. The VSF protein comprises several subunits, in which the subunits are associated with each other by covalent or non-covalent bonding, thereby forming a macromolecule having a molecular weight of over about 100 kDa.

Through the measurement of molecular weight of the VSF protein using Centricon™, the VSF protein was identified to be present as a macromolecule of over 100 kDa in a naturally occurring state, which is a minimum unit capable of displaying antiviral activity (Example 9), and the highest antiviral activity of the VSF protein was found in a fraction of over 500 kDa.

When analyzing a 163 kDa fraction, purified by using a FPLC (Fast Flow Performance Liquid Chromatography) column, by SDS-PAGE, the resulting protein pattern is identical to major bands as shown in FIG. 1, found in active fractions obtained by chromatography using three different columns (hydroxyapatite column, Protein A agarose column, and Blue Sepharose column). Therefore, an active form of the VSF protein is believed to have a molecular weight

of 163 kDa.

On the other hand, when analyzing fractions obtained by performing open column chromatography (using hydroxyapatite column, Protein A agarose column, and Blue Sepharose column) and FPLC, and sucrose gradient centrifugation, by SDS-PAGE, four major bands of below 100 kDa were found, which are H (about 55kDa), L1 (about 30kDa), L2 (about 30kDa) and L3 (about 25 kDa). As a result of measuring density of the bands by densitometry, ratio of H: L1: L2: L3 was 1: 0.5-1: 0.5-1: 0.5-2. Subunits of the VSF protein of the present invention are believed to associate by relatively strong bonding.

As a result of Western blotting using anti-mouse IgG(H+L) antibody and anti-mouse Fab-specific antibody, the four major bands as shown in FIG. 1 were detected. When using anti-mouse gamma heavy chain-specific antibody, only the H band (about 55 kDa) among the four major bands was detected. When using anti-mouse κ-light chain-specific antibody, three bands (L1, L2 and L3) of the four major bands were detected.

3. H and L3 subunit of the VSF protein have DNA sequences and amino acid sequences designated as SEQ ID NOs 1 to 4, as follows.

SEQ ID NO: 1: DNA sequence of H subunit of VSF protein

ATGGGATGGAGCTGGATCTTTCTCTTCCTTCTGTCAGTAACTGCAGGTGTCCACTCT
GAGATCCAGCTGCAGCAGTCTGGAGCTGAGCTGGTGAAGCCTGGGGCTTCAGTGA
AGATATCCTGCAAGGCTTCTGGTTACTCATTCACTGGCTACAACATGAACTGGGTGA
AGCAGAGCCATGGAAAGAGCCTTGAGTGGATTGGAAATATTGATCCTTACTATGGT
AGTACTACCTACAATCAGAAGTTCAAGGGCAAGGCCACATTGACTGTAGACAAATC
TTCCAGCACAGCCTACATGCAGCTCAACAGCCTGACATCTGAGGACTCTGCAGTCT
ATTACTGTGCAAGAGAGACTGGGACGAGGGCTATGGACTACTGGGGTCAAGGAAC
CTCAGTCACCGTCTCCTCAGCTACAACAACAGCCCCATCTGTCTATCCCTTGGTCCC
TGGCTGCAGTGACACATCTGGATCCTCGGTGACACTGGGATGCCTTGTCAAAGGCT

ACTTCCCTGAGCCGGTAACTGTAAAATGGAACTATGGAGCCCTGTCCAGCGGTGTG
CGCACAGTCTCATCTGTCCTGCAGTCTGGGTTCTATTCCCTCAGCAGCTTGGTGACT
GTACCCTCCAGCACCTGGCCCAGCCAGACTGTCATCTGCAACGTAGCCCACCCAGC
CAGCAAGACTGAGTTGATCAAGAGAATCGAGCCTAGAATACCCAAGCCCAGTACC
CCCCCAGGTTCTTCATGCCCACCTGGTAACATCTTGGGTGGACCATCCGTCTTCATC
TTCCCCCCAAAGCCCAAGGATGCACTCATGATCTCCCTAACCCCCAAGGTTACGTG
TGTGGTGGTGGATGTGAGCGAGGATGACCCAGATGTCCATGTCAGCTGGTTTGTGG
ACAACAAAGAAGTACACACAGCCTGGACACAGCCCCGTGAAGCTCAGTACAACA
GTACCTTCCGAGTGGTCAGTGCCCTCCCCATCCAGCACCAGGACTGGATGAGGGG
CAAGGAGTTCAAATGCAAGGTCAACAACAAAGCCCTCCCAGCCCCCATCGAGAGA
ACCATCTCAAAACCCAAAGGAAGAGCCCAGACACCTCAAGTATACACCATACCCC
CACCTCGTGAACAAATGTCCAAGAAGAAGGTTAGTCTGACCTGCCTGGTCACCAA
CTTCTTCTCTGAAGCCATCAGTGTGGAGTGGGAAAGGAACGGAGAACTGGAGCAG
GATTACAAGAACACTCCACCCATCCTGGACTCAGATGGGACCTACTTCCTCTACAG
CAAGCTCACTGTGGATACAGACAGTTGGTTGCAAGGAGAAATTTTTACCTGCTCCG
TGGTGCATGAGGCTCTCCATAACCACCACACACAGAAGAACCTGTCTCGCTCCCCT
GGTAAA

SEQ ID NO: 2: Amino acid sequence of H subunit of VSF protein

MGWSWIFLFLLSVTAGVHSEIQLQQSGAELVKPGASVKISCKASGYSFTGYNMNWVK
QSHGKSLEWIGNIDPYYGSTTYNQKFKGKATLTVDKSSSTAYMQLNSLTSEDSAVYYC
ARETGTRAMDYWGQGTSVTVSSATTTAPSVYPLVPGCSDTSGSSVTLGCLVKGYFPEP
VTVKWNYGALSSGVRTVSSVLQSGFYSLSSLVTVPSSTWPSQTVICNVAHPASKTELIK
RIEPRIPKPSTPPGSSCPPGNILGGPSVFIFPPKPKDALMISLTPKVTCVVVDVSEDDPDV
HVSWFVDNKEVHTAWTQPREAQYNSTFRVVSALPIQHQDWMRGKEFKCKVNNKALP
APIERTISKPKGRAQTPQVYTIPPPREQMSKKKVSLTCLVTNFFSEAISVEWERNGELEQ

DYKNTPPILDSDGTYFLYSKLTVDTDSWLQGEIFTCSVVHEALHNHHTQKNLSRSPGK

SEQ ID NO: 3: DNA sequence of L3 subunit of VSF protein

ATGAGTGTGCCCACTCAGGTCCTGGGGTTGCTGCTGCTGTGGCTTACAGGTGCCAG
ATGTGACATCCAGATGACTCAGTCTCCAGCCTCCCTATCTGCATCTGTGGGAGAAA
CTGTCACCATGACATGTCGAGCAAGTGAGAATATTTACAGTAATTTAGCATGGTATC
AGCAGAAACAGGGAAAATCTCCTCAGCTCCTGGTCTATGTTGCAACAAACTTAGCA
GATGGTGTGCCATCAAGGTTCAGTGGCAGTGGATCAGGCACACAGTTTTCTCTGAA
GATCAACAGCCTGCAGCCTGAAGATTTTGGGAGTTATTACTGTCAACATTTTTATGG
TTCTCCTCGGACGTTCGGTGGAGGCACCAAGCTGGAAATCAAACGGGCTGATGCT
GCACCAACTGTATCCATCTTCCCACCATCCAGTGAGCAGTTAACATCTGGAGGTGC
CTCAGTCGTGTGCTTCTTGAACAACTTCTACCCCAGAGACATCAATGTCAAGTGGA
AGATTGATGGCAGTGAACGACAAAATGGTGTCCTGAACAGTTGGACTGATCAGGA
CAGCAAAGACAGCACCTACAGCATGAGCAGCACCCTCACATTGACCAAGGACGAG
TATGAACGACATAACAACTATACCTGTGAGGCCACTCACAAGACATCAACTTCACC
CATCGTCAAGAGCTTCAACAGGAATGAGTGT

SEQ ID NO: 4: Amino acid sequence of L3 subunit of VSF protein

MSVPTQVLGLLLLWLTGARCDIQMTQSPASLSASVGETVTMTCRASENIYSNLAWYQ
QKQGKSPQLLVYVATNLADGVPSRFSGSGSGTQFSLKINSLQPEDFGSYYCQHFYGSPR
TFGGGTKLEIKRADAAPTVSIFPPSSEQLTSGGASVVCFLNNFYPRDINVKWKIDGSER
QNGVLNSWTDQDSKDSTYSMSSTLTLTKDEYERHNNYTCEATHKTSTSPIVKSFNRNE
C

As a result of BLAST search for amino acid homology of a N-terminal region (137 amino acids) of the H subunit and a N-terminal region (127 amino acids) of the L3 subunit of the

VSF protein, the N-terminal region of the H subunit was found to have homology with the known amino acid sequences of the variable (V) region (score: 222) of Ig heavy chain precursor, and the Ig gamma1 heavy chain (score: 217), in addition to with the unidentified protein for MGC:19223 (score: 216). The N-terminal region of the L3 subunit has amino acid homology with Ig kappa chain (score: 206) and the V region of a Ig kappa chain precursor (score: 199), in addition to with the unidentified protein for MGC:28604 (score: 199).

4. When subjecting the VSF protein to immunoprecipitation and immunoneutralization using sheep anti-mouse IgG(H+L), antiviral activity of the VSF protein was not changed. In detail, after incubating the VSF protein of the present invention along with 100 μg of sheep anti-mouse IgG(H+L) at 4°C overnight, and centrifuging the reaction mixture at 1000×g for 10 min, and analyzing the supernatant by a virus inhibition test (VIT), the antiviral activity of the VSF protein was found not to be changed.

5. The VSF protein did not show a band shift on a SDS-PAGE gel even after treatment of endoglycosidase. This result indicates that the VSF protein is nonglycosylated, or glycosylated at a very low level.

6. The VSF protein was evaluated for sensitivity to various proteinases. In case of being treated with papain, the heavy chain of IgG and the H subunit of the VSF protein showed different reactivity to anti-gamma. In case of being treated with pepsin, the VSF protein and IgG showed different reactivities to anti-Fab. Also, all of H, L1, L2 and L3 subunits of the VSF protein were found to be sensitive to proteinase K. When being treated with trypsin, both of heavy and light chains of mouse IgG showed weak resistance to trypsin, while, in case of the VSF protein, the H subunit was very sensitive to trypsin and thus destroyed, but the L1, L2 and L3 subunits were minimally degraded by trypsin cleavage. Further, the VSF protein was not cleaved by cathepsin.

In another aspect of the present invention, there is provided a hybridoma prepared by stimulating an immune cell with a variant of encephalomyocarditis virus, EMC-DV, isolating the

immune cell and fusing the immune cell with a tumor cell, and a method of preparing such a hybridoma.

In a further aspect of the present invention, there is provided a method of preparing a VSF protein, comprising culturing a hybridoma prepared as described above, and isolating a VSF protein from the culture fluid of the hybridoma.

In a still further aspect of the present invention, there is provided a method of preparing a VSF protein, comprising injecting a hybridoma prepared as described above into an animal, and isolating a VSF protein from an ascitic fluid obtained from the animal.

The virus, used to obtain stimulated immune cells, is administered into a host by intraperitoneal, intravenous, intramuscular, intraocular or subcutaneous injection, after being properly diluted in a suitable solvent, for example, a physiological saline solution, to prevent induction of physiological disorder and apoptosis in the host. The virus is administered into the host at an amount suitable for inducing the immune response in the host. The preferred dosage of the virus is from 10^3 to 10^9 pfu per animal. Administration frequency is typically 2 to 4 at intervals of 1 to 2 weeks. For example, in case of administering the virus four times, the first to third administrations are performed by intraperitoneal injection, and the final administration is by intravenous injection.

3 to 4 days after the final administration, the animal host infected with a virus is anatomized, and then splenocytes are isolated from the excised spleen and used as immune cells. The tumor cells fused with the immune cells include myeloma cells, including mouse-derived cells which are exemplified by p3/x63-Ag8, p3-U1, NS-1, MPC-11, SP-2/0, F0, P3x63 Ag8, V653 and S194, and rat-derived cells such as R-210.

The immune cells infected with a variant of encephalomyocarditis virus, EMC-DV, may be fused with tumor cells by the conventional method, and, from the resulting hybridoma cells, a hybridoma having the highest virus inhibitory effect is selected. Such a hybridoma may be prepared by the conventional immunochemical technique, in which mice and rats may be used.

For example, fusion of immune cells with tumor cells may be achieved by the conventionally known method, which is exemplified by the Koehler & Milstein's method (Koehler et al., Nature 256, 495-497, 1975) that is generally used, and electrofusion using electric pulse. Lymphocytes and a myeloma cell line are mixed at a ratio commonly used in the art, subjected to cell fusion using a FCS-free culture medium containing polyethyleneglycol, which is generally used in the art, and cultured in FCS-containing HAT selection medium to select fused cells (hybridoma cells). In connection with this, the present invention provides a hybridoma producing a VSF protein. The hybridoma of the present invention was deposited with an international depository authority, KCLRF (Korean Cell Line Research Foundation) on Dec. 21, 2001 under the provisions of the Budapest Treaty and has been assigned accession number KCLRF-BP-00052.

The hybridoma can be subcultured by the conventional culturing method, and cryo-preserved according to intended use. VSF protein may be recovered by collecting the culture fluids after culturing the hybridoma by the conventional method, or by injecting the hybridoma into a mammal and then obtaining ascitic fluids from the abdominal cavity of the mammal. VSF protein contained in culture fluids or ascitic fluids may be purified by the conventional method, including salting out, ion exchange and gel filtration chromatography, and affinity column chromatography. Preferably, the VSF protein may be purified by chromatography using a Blue Sepharose column or hydroxyapatite resin column, affinity chromatography using a Protein A agarose column, FPLC, or sucrose gradient centrifugation.

In detail, the VSF protein may be isolated by Blue Sepharose column chromatography, as follows.

The culture fluid of the hybridoma of the present invention or the supernatant of the ascitic fluid is passed through a Blue Sepharose column (Blue Sepharose CL-6B resin column) at a flow rate of 0.1-1.0 ml/min.

Proteins not bound to the column are eliminated by washing the column with sodium phosphate buffer (pH 7.0-7.5) containing 10-60 mM NaCl. Then, sodium phosphate buffer (pH

7.0-7.5) containing 100-300 mM NaCl is added to the column to elute VSF protein, thereby generating active fractions containing VSF protein. Preferably, the culture fluid of the hybridoma or the supernatant of the ascitic fluid is passed through a Blue Sepharose column sufficiently equilibrated with 20 mM sodium phosphate buffer (pH 7.2) at a flow rate of 0.5 ml/min. To eliminate proteins not bound to the column, the column is sufficiently washed with 20 mM sodium phosphate buffer (pH 7.2) containing 50 mM NaCl, where the washing continues until absorbance at 280 nm of the flow through washing buffer falls to a background level.

VSF protein bound to the column is eluted by adding 50 mM sodium phosphate buffer (pH 7.2) containing 200 mM NaCl to the column, thereby giving active fractions. The active fractions are desalted and concentrated using a concentrator.

In addition, the VSF protein according to the present invention may be isolated by chromatography using a Protein A agarose column, as follows. The culture fluid of the hybridoma of the present invention or the supernatant of the ascitic fluid is passed through a Protein A agarose column at a flow rate of 0.1-1.0 ml/min. Proteins not bound to the column are eliminated by washing the column with Tris buffer. Then, acetic acid buffer (pH 4.0-5.0) is added to the column to elute VSF protein, thereby generating active fractions containing the VSF protein. The eluted protein is neutralized with 0.1-1.0 M sodium phosphate buffer. Preferably, the culture fluid of the hybridoma or the supernatant of the ascitic fluid is passed through a Protein A agarose column at a flow rate of 0.5 ml/min. To eliminate proteins not bound to the column, the column is sufficiently washed with 0.1 M Tris buffer, where the washing continues until absorbance at 280 nm of the flow through washing buffer falls to a background level. VSF protein bound to the column is eluted by adding acetic acid buffer (pH 4.3) to the column, thereby giving active fractions. The eluted protein is neutralized with 0.5 M sodium phosphate buffer (pH 7.7).

In addition, the VSF protein according to the present invention may be isolated by chromatography using a hydroxyapatite resin column, as follows. The culture fluid of the hybridoma of the present invention or the supernatant of the ascitic fluid is passed through a

column filled with hydroxyapatite resin at a flow rate of 0.5-1.5 ml/min. Proteins not bound to the column are eliminated by washing the column with a phosphate-buffered solution. Then, 50-150 mM potassium phosphate buffer (pH 7.0-7.5) is added to the column to elute VSF protein, thereby generating active fractions containing the VSF protein. The collected active fractions are desalted and concentrated using a concentrator. Preferably, a column filled with 15 ml of hydroxyapatite resin is equilibrated with 2 mM phosphate buffer (pH 7.2) containing 1 mM 2-mercaptoethanol and 0.5 mM EDTA. A sample according to the present invention flows through the column, and then the column is sufficiently washed with the equilibrating buffer. VSF protein bound to the column is eluted by adding column buffer containing 100 mM potassium phosphate (pH 7.2) to the column, thereby giving active fractions. The collected active fractions are desalted and concentrated until a final concentration of potassium phosphate (pH 7.2) is 2 mM, using a concentrator.

The present invention provides a pharmaceutical composition for prevention and treatment of viral infections in an animal, comprising a therapeutically or preventively effective amount of a VSF protein and a pharmaceutically acceptable carrier, and a method of preventing or treating viral infections, comprising administration of such a pharmaceutical composition.

The therapeutically or preventively effective amount of the VSF protein may be properly determined according to administration subjects, and subjects' age and pathogenic states, and difference between subjects. For example, adult dosage may be about 5 μg-1 mg/kg body weight. In case of oral administration, the pharmaceutically acceptable carrier may include binders, lubricants, disintegrators, excipients, solubilizers, dispersing agents, stabilizers, suspending agents, coloring agents and perfumes. In case of injection preparations, the pharmaceutically acceptable carrier may include buffering agents, preserving agents, analgesics, solubilizers, isotonic agents and stabilizers. In case of preparations for local administration, the pharmaceutically acceptable carrier may include bases, excipients, lubricants and preserving agents. The pharmaceutical composition may be formulated into a variety of dosage forms in combination with the aforementioned pharmaceutically acceptable carrier. For example, for oral administration, the

pharmaceutical composition may be formulated into tablets, troches, capsules, elixirs, suspensions, syrups or wafers. For injection preparations, the pharmaceutical composition may be formulated into a unit dosage form, such as a multidose container or an ampule as a single-dose dosage form.

The antiviral pharmaceutical composition according to the present invention may be administered orally, or parenterally, i.e., by intravenous, subcutaneous, intranasal or intraperitoneal administration, to humans or animals. The oral administration includes sublingual application. The parenteral administration includes injection methods such as subcutaneous, intramuscular or intravenous injection, and drip injection.

In addition, the pharmaceutical composition according to the present invention may be formulated into other forms according to the conventional method.

The present invention will be explained in more detail with reference to the following an example in conjunction with the accompanying drawings. However, the following example is provided only to illustrate the present invention, and the present invention is not limited to the example.

EXAMPLE 1: Preparation of hybridomas producing VSF protein

The EMCV variant, EMC-DV, was intraperitoneally injected into five 8 week-old BALB/c mice (female) at an amount of 5×10^4 pfu (plaque forming unit)/mouse. After 7 days, 10^7 pfu of EMC-DV was intravenously injected. After 4 days, mice were sacrificed to excise spleens. The splenocytes obtained from the excised spleens were fused with myeloma cells (V653) using polyethylene glycol.

The resulting fused cell clones were subjected to a virus inhibition test (VIT) to screen cell lines having high antiviral activity. VIT was performed using DMEM (Gibco) containing 2% FCS. 50 μl of culture fluid serially diluted over four times was aliquotted into each well of a 96-well plate. 5×10^4 L cells (murine fibroblasts) were put into each well of the plate. After

incubation for 1 hr, 100 pfu of EMCV was added to each well, to make a total volume of 200 μl, followed by incubation at 37°C for 48 hrs. After incubation, the culture medium was removed, and then 150 μl of 10% formaldehyde was added to each well, and the plate was incubated at room temperature for 10 min to allow fixation of L cells. 50 μl of 1% crystal violet solution was added to each well, followed by incubation at room temperature for 10 min to allow staining. Thereafter, the added solutions were removed, and the plate was lightly washed with water to eliminate background stain. Cellular viability was evaluated by analyzing stained degree of the cells.

When viral proliferation is suppressed, all cells survive, forming a uniform layer, and are stained with crystal violet, giving a uniformly stained layer. In contrast, when cells are lyzed by viral infection, the cells are detached, thereby yielding rarely stained layer when being stained.

In addition, inhibitory effect of the VSF protein against viruses was analyzed by investigating cellular viability using MTS ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium], inner salt, Promega), which is used for measurement of metabolic rates of cells. According to the protocol recommended by the manufacturing company, appropriate cells were plated into a 96-well plate at a density of 10^4 cells, and treated with the culture fluid containing VSF protein of various concentrations, as well as with a virus. After incubation for a predetermined time according to life cycle of the virus, the cells were treated with 20 μl of a MTS solution, and then absorbance at 490 nm was measured.

The higher cellular viability is, the stronger absorbance at 490 nm is. Cellular viability was calculated from the measured absorbance values.

As a result, a hybridoma cell line 4D1B having excellent antiviral activity was obtained, and deposited in an international depository authority, KCLRF (Korean Cell Line Research Foundation) on Dec. 21, 2001 under the provisions of the Budapest Treaty and has been assigned accession number KCLRF-BP-00052.

EXAMPLE 2: Production of ascitic fluids

The hybridoma 4D1B (accession number KCLRF-BP-00052) prepared in Example 1 was inoculated in DMEM containing 2% FCS, and incubated at 37°C under 5% CO_2. 1×10^7-2×10^7 cells of the cultured hybridma were intraperitoneally injected into mice. After about 2 weeks, ascitic fluids were collected from the swelled abdominal cavities of mice, and centrifuged at 1500×g. The resulting supernatants were aliquotted to a small volume, as described above, and stored at -70°C until use.

EXAMPLE 3: Sensitivity of VSF protein to proteinase

The VSF protein according to the present invention was evaluated for sensitivity to proteinase. In this test, proteinase K was used, which has specific activity of 30 units/mg. The hybridoma 4D1B (accession number KCLRF-BP-00052) prepared in Example 1 was inoculated in SFM (serum-free media, Gibco), and incubated at 37°C under 5% CO_2. Proteinase K was added to the culture fluid at an amount of 1, 2 and 4 mg/ml, followed by incubation at 37°C for 1 hr. Separately, trypsin, having specific activity of 10,000 BAEE units/mg protein, was added to the culture fluid at an amount of 1 mg/ml, followed by incubation for 3 hrs. Antiviral activity of VSF protein contained in the culture fluid was analyzed by VIT and MTS assay as described in Example 1.

As a result, even when treated with a minimum amount of proteinase K, VSF protein lost its antiviral activity. In contrast, in case of being treated with trypsin, VSF protein was found to retain weak antiviral activity. These results indicate that all or some subunits of the VSF protein are very sensitive to proteinase K.

EXAMPLE 4: Evaluation of the hybridoma for expression of cytokines by RT-PCR

The hybridoma 4D1B (accession number KCLRF-BP-00052) secreting VSF protein, prepared in Example 1, was evaluated for expression of specific cytokines by RT-PCR. RT-PCR was carried out to the hybridoma 4D1B and the negative controls which were the splenocytes from mice used in preparing fused cells in Example 1 and a hybridoma not having antiviral activity, as follows.

Total RNA was isolated from the above cells using Trizol reagent (Gibco-BRL). Chemical reagents used in RT-PCR were purchased from Promega. To a sterilized microtube, 5 μg of total RNA, 8 μl of 5× reverse transcriptase buffer, 8 μl of 10mM dNTP, 100 pmole of oligo(dT), 40 units of RNasin, and 200 units of MMLV reverse transcriptase were added, and total volume of the mixture was adjusted to 40 μl using DEPC(diethyl pyrocarbonate)-treated water. Then, the mixture was incubated at 42°C for 1 hr, and then at 95°C for 3 min, thus yielding a cDNA sample.

The cDNA sample was subjected to PCR using a primer set capable of amplifying a specific cytokine. The primer sets (synthesized by Bioneer, Korea) are listed in Table 1, below, and can amplify cytokines including IL, IFN, TNF, TGF, CSF and chemokines. To a premix tube, 1 μl of a cDNA sample, 10 pmole of forward primer and 10 pmole of reverse primer were added, and total volume of the mixture was adjusted to 20 μl using sterilized water. PCR was carried out under conditions of denaturation at 94°C for 3 min, and 45 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min, followed by final extension at 72°C for 3 min.

TABLE 1

Primers for RT-PCR

Primer name	Sequence	SEQ ID NO	Primer name	Sequence	SEQ ID NO
IL-1aF	cagcaccttacacctaccagag	5	IL-1aR	ctggaagtctgtcatagagg	6
IL-1bF	gcagctatggcaactgttcc	7	IL-1bR	ggaagacacagattccatggtg	8
IL-2F	atgtacagcatgcagctcgc	9	IL-2R	gagggcttgttgagatgatgc	10
IL-3F	atggttcttgccagctctaccac	11	IL-3R	ttaacattccacggttccacgg	12
IL-4F	atgggtctcaacccccagctag	13	IL-4R	gatgctctttaggctttccagg	14
IL-5F	cagagtcatgagaaggatgc	15	IL-5R	tcagccttccattgcccactctg	16
IL-6F	gaagttcctctctgcaagag	17	IL-6R	ctaggtttgccgagtagatctc	18
IL-7F	cgcagaccatgttccatgtttc	19	IL-7R	ggaggttgttactacatgtcctg	20
IL-9F	ggtgacatacatccttgcctc	21	IL-9R	tcatggtcggcttttctgcc	22
IL-10F	atgcctggctcagcactgctatg	23	IL-10R	catggccttgtagacaccttg	24
IL-11F	atgaactgtgtttgtcgcctgg	25	IL-11R	tcccgagtcacagtcgagtc	26
IL-12aF	atgtgtcaatcacgctacctcctc	27	IL-12aR	tcaggcggagctcagatagc	28
IL-12bF	atgtgtcctcagaagctaacc	29	IL-12bR	atcctaggatcggaccctgc	30
IL-13F	ttcatggcgctctgggtgac	31	IL-13R	tcattagaagggccgtggc	32
IL-15F	gaggaatacatccatctcgtgc	33	IL-15R	cagtcaggacgtgttgatgaac	34
IL-16F	cacggttcacagagtgtttcc	35	IL-16R	ctatgagtctgcagaagctg	36
IL-17F	atgagtccagggagagcttc	37	IL-17R	ttaggctgcctggcggacaatc	38
IL-18F	atggctgccatgtcagaagac	39	IL-18R	gcatcatcttccttttggcaagc	40
G-CSFF	atggctcaactttctgcccag	41	G-CSFR	ctaggccaagtggtgcagagc	42
GM-CSFF	tgaggaggatgtggctgcag	43	GM-CSFR	gcttcctcatttttggactgg	44
TNF-αF	atgagcacagaaagcatgatccg	45	TNF-αR	cttcacagagcaatgactcc	46
TNF-βF	atgacactgctcggccgtct	47	TNF-βR	gaatctacagtgcaaaggctcc	48
IFN-αF	cataatggctaggccctttgc	49	IFN-αR	tcactccttctcctcactcag	50
IFN-βF	atgaacaacaggtggatcctcc	51	IFN-βR	ctgacaggtcttcagttttgg	52
IFN-γF	atgaacgctacacactgcatc	53	IFN-γR	tcagcagcgactccttttcc	54
TGF-βF	gcgctcactgctcttgtgac	55	TGF-βR	ttcagctgcacttgcaggag	56
RANTESF	atgaagatctctgcagctgcc	57	RANTESF	cctctatcctagctcatctcc	58
MIP-1αF	atgaaggtctccaccactgc	59	MIP-1αR	ctcaggcattcagttccagg	60
MIP-1βF	atgaagctctgcgtgtctgc	61	MIP-1βR	ctggagctgctcagttcaac	62
MIP-1γF	atgaagccttttcatactgccc	63	MIP-1γR	gttattgtttgtaggtccgtgg	64
MCP-1F	atgcaggtccctgtcatgcttc	65	MCP-1R	ctagttcactgtcacactggtc	66
MCP-3F	atgaggatctctgccacgcttc	67	MCP-3R	cttcaaggctttggagttgggg	68
IP-10F	atgaacccaagtgctgccgt	69	IP-10R	cagttaaggagccctttttagacc	70
LymphotactinF	atgagacttctcctcctgac	71	LymphotactinR	ctgttacccagtcagggtta	72
GapdhF	tgatgggtgtgaaccacgag	73	GapdR	cttactccttggaggccatg	74

As a result, it was found that there is no expression of specific cytokines in the hybridoma secreting the VSF protein of the present invention, and this result was reproduced in repeated experiments (Table 2, O: expression of cytokine). This result indicates that the VSF protein of the present invention does not comprise any conventionally known cytokine, and thus may be a new protein.

TABLE 2

RT-PCR result for cytokine expression

No.	Cytokine	bp	Splenocytes	Control hybridoma	Hybridoma of the present invention
1	IL-1alpha	461	O		
2	IL-1beta	813	O		
3	IL-2	505			
4	IL-3	501			
5	IL-4	402	O		
6	IL-5	409			
7	IL-6	634	O		
8	IL-7	497	O		
9	IL-9	430	O		
10	IL-10	474	O	O	O
11	IL-11	607			
12	IL-12a	648			
13	IL-12b	1011			
14	IL-13	402		O	O
15	IL-15	469	O	O	O
16	IL-16	574	O	O	O
17	IL-17	477			
18	IL-18	497	O	O	O
19	G-CSF	627			
20	GM-CSF	440			
21	TNF-α	710	O		
22	TNF-β	613	O		
23	IFN-α	577			
24	IFN-β	560			
25	IFN-γ	468	O		
26	TGF-β	516	O	O	O
27	RANTES	284	O	O	
28	MIP-1α	280	O		
29	MIP-1β	289	O		
30	MIP-1γ	370	O		
31	MCP-1	447	O		
32	MCP-3	296		O	
33	IP-10	300	O	O	
34	Lymphotactin	348	O		
35	Gapdh	615	O	O	O

EXAMPLE 5: Purification of VSF protein by Blue Sepharose column chromatography

The hybridoma 4D1B (accession number KCLRF-BP-00052) was cultured in 20% FCS-containing DMEM, and then SFM (serum-free medium) or PFM (protein-free medium). The

medium was exchanged with a fresh medium at intervals of 2 days. The culture fluids were collected, and centrifuged at 1500×g to remove cell debris.

The supernatant was passed through a Blue Sepharose column at a flow rate of 0.5 ml/min. To eliminate proteins not bound to the column, the column was sufficiently washed with 20 mM sodium phosphate buffer (pH 7.2) containing 50 mM NaCl. Then, 50 mM sodium phosphate buffer (pH 7.2) containing 200 mM NaCl was added to the column, and active fractions containing VSF protein were collected.

EXAMPLE 6: Purification of VSF protein by Protein A affinity column chromatography

The hybridoma 4D1B (accession number KCLRF-BP-00052) was cultured in 20% FCS-containing DMEM, and then SFM (serum-free medium) or PFM (protein-free medium). The medium was exchanged with a fresh medium at intervals of 2 days. The culture fluids were collected, and centrifuged at 1500×g to remove cell debris.

An affinity column was prepared using Protein A agarose bead (Sigma, Cat. #P-2545). The supernatant was passed through the affinity column at a flow rate of 0.5 ml/min. Active fractions containing VSF protein were collected by adding acetic acid buffer (pH 4.3) to the column.

EXAMPLE 7: Purification of VSF protein by hydroxyapatite column chromatography

The hybridoma 4D1B (accession number KCLRF-BP-00052) was cultured in 20% FCS-containing DMEM, and then SFM (serum-free medium) or PFM (protein-free medium). The medium was exchanged with a fresh medium at intervals of 2 days. The culture fluids were collected, and centrifuged at 1500×g to remove cell debris.

A column filled with hydroxyapatite resin was equilibrated with 2 mM phosphate buffer (pH 7.2) containing 1 mM 2-mercaptoethanol and 0.5 mM EDTA. The supernatant was passed

through the column at a flow rate of 0.5 ml/min. After sufficiently washing the column with the aforementioned buffer, active fractions containing VSF protein were collected by adding 100 mM potassium phosphate (pH 7.2) to the column.

EXAMPLE 8: Analysis of active fractions containing VSF protein by SDS-PAGE

The active fractions containing VSF protein, prepared in Examples 5 to 7, was analyzed by SDS-PAGE and Western blotting, in which proteins were detected by staining with Coommassie Blue and immunostaining using AP-conjugated anti-mouse IgG antibody. 5 μl of each active fraction was mixed with 2× SDS sample buffer, boiled for 5 min, cooled, and loaded onto a 12% SDS-polyacrylamide gel.

After electrophoresis, proteins separated on the gel were identified by staining with Coommassie Blue dye, and then destaining using an acetic acid-methanol mixture to remove background staining. Separately, to detect proteins by Western blotting, each active fraction was separated on a SDS-polyacrylamide gel according to the same method as described above, and transferred onto a nitrocellulose membrane. The membrane was washed with distilled water and dried. After being blocked for 1 hr 30 min in 5% skim milk dissolved in Blotto (TBS (tris-buffered saline)), and washed with TBS and dried, the membrane was reacted with alkaline phosphatase (AP)-conjugated goat anti-mouse IgG for 1 hr. The membrane was washed three times with TBS containing Tween 20 at intervals of 10 min, and then with AP buffer (100 mM Tris buffer containing 5 mM $MgCl_2$, pH 9.5) for 10 min. Thereafter, the membrane was developed using 10 ml AP buffer supplemented with 66 μl of a NBT stock solution prepared by dissolving NBT (nitroblue tetrazolium) at a concentration of 50 mg/ml in 70% DMF (dimethylformamide), and 30 μl of a BCIP stock solution prepared by dissolving BCIP (bromochloroindolyl phosphate) at a concentration of 50 mg/ml in 100% DMF. When the development reached a suitable strength, the blot was washed with distilled water several times to stop the color reaction.

As shown in FIG. 1, major bands of the active fractions obtained using three different columns were found to be almost identical. 3 major bands were present at identical positions in three kinds of active fractions, and one band among them was identified to be a doublet upon magnifying the gel image. One band (about 55 kDa), two bands (about 30 kDa) and one band (about 25 kDa) were designated 'H', 'L1' and 'L2', and 'L3', respectively. Herein, in case of the active fractions obtained using a Protein A agarose resin, an antibody-like molecule was found to be isolated. It is believed that the molecule is not just an antibody to a specific molecule, but a member of the immunoglobulin superfamily, or that a part of an antibody is required for action of the VSF protein.

EXAMPLE 9: Determination of molecular weight of VSF protein using Centricon™

Molecular weight of the VSF protein according to the present invention was determined using Centricon membranes (Amicon) with 30, 50, 100 and 500 kDa pores. The active fractions prepared in Examples 5 to 7 were loaded onto a Centricon membrane with 30 kDa pore, followed by centrifugation at 5000×g. Fluids passed through the Centricon membrane with 30 kDa pore were stored, and the remaining fraction solutions were subjected to a Centricon membrane with a larger size pore, where the Centricon membranes with 50, 100 and 500 kDa pores were used sequentially. Fluids passed through each Centricon membrane were analyzed for antiviral activity according to the same method as in Example 1, as well as for protein distribution by SDS-PAGE.

As shown in Table 3, below, antiviral activity was found mainly in the fractions containing over 500 kDa proteins and 100 to 500 kDa proteins, while weak antiviral activity was found in the fraction containing less than 100 kDa proteins. These results were identical to the SDS-PAGE results as shown in FIG. 2. It was shown that major protein did not pass through Centricon of 500 kDa pore with a relatively small portion being present in 100 to 500 kDa pore. These results indicates that the VSF protein is actually present at a large complex form with a

molecular weight of at least over 100 kDa, which is a minimum unit capable of displaying antiviral activity. The conventional CAF was known to have a molecular weight of less than 30 kDa, when isolated according to the method as described above. Therefore, it is believed that the VSF protein of the present invention is completely different from CAF. Moreover, with consideration of the fact that most of the conventionally known antiviral cytokines have a molecular weight of less than 100 kDa, it appears that the VSF protein does not comprise any of the conventionally known antiviral cytokines.

TABLE 3

Assay result for antiviral activity of fluids passed through Centricon™

Pore size of Centricon™	VIT#	Total protein amount (mg/ml)
> 500 kDa	1,024	0.32
100-500 kDa	256	0.2
50-100 kDa	1	0.14

VIT#: Virus Inhibition Test: maximum dilution times capable of detecting antiviral activity

EXAMPLE 10: Evaluation for antiviral activity of VSF protein against EMCV and Mengo virus

The culture fluid of the hybridoma 4D1B (accession number KCLRF-BP-00052), and ascitic fluids prepared in Example 2, were evaluated for antiviral activity against EMCV and Mengo virus according to the same method as in Example 1.

As shown in Table 4, below, the VSF protein was found to strongly suppress proliferation of EMCV and Mengo virus. Even when diluted over 2048 times, VSF protein contained in the culture fluid retained antiviral activity, while VSF protein contained in the ascitic fluids had antiviral activity even when diluted one million times. Also, these results were confirmed by MTS assay

(FIG. 3).

The conventionally known CAF was reported to retain antiviral activity only up to a 10-fold dilution (Walker CM et al., Immunology, 66, 628-630 (1989), Carl EM et al., PNAS, 92, 2308-2312 (1995)), whereas the VSF protein of the present invention was demonstrated to have very strong antiviral activity. In addition, the VSF protein showed no cytotoxic activity even when cells were treated with the highest concentration of the VSF protein, indicating that antiviral activity of the VSF protein is not based on apoptosis, and the VSF protein has very low cytotoxic activity.

TABLE 4

Inhibitory effect of VSF protein on proliferation of EMCV and Mengo virus

	Culture fluid	Ascitic fluid A	Ascitic fluid B	Ascitic fluid C	Ascitic fluid D
VIT#	2,048	1,048,576	1,048,576	4,194,304	1,048,576

VIT#: Virus Inhibition Test: maximum dilution times capable of detecting antiviral activity

EXAMPLE 11: Evaluation for antiviral activity of VSF protein against influenza virus

The culture fluid of the hybridoma 4D1B (accession number KCLRF-BP-00052), and ascitic fluids prepared in Example 2, were evaluated for antiviral activity against influenza virus, as follows. After incubation for 8 days, embryonated chicken eggs were inoculated with influenza virus. First, after selecting areas not having blood vessels of the eggs as inoculation sites, the surface of the shell on the inoculation sites was disinfected. After punching the shell containing the shell membrane to make a small hole, influenza virus was inoculated into the allantoic cavity through the hole using a microinjector. Then, the hole was sealed with drops of melted wax. Herein, 1 HAU (hemagglutination unit) of influenza virus was inoculated, and 10-200 μl of the culture fluid of the hybridoma or the ascitic fluid was inoculated. After incubation for 2 days at

34°C, some of the inoculated eggs were placed at 4°C overnight, and a part of the shell was broken to collect the allantoic fluid. The remaining eggs were further incubated for 3 more days, and the shell was removed and viability of the embryos was investigated. The collected allantoic fluids were subjected to hemagglutination assay. The allantoic fluids were serially diluted with phosphate-buffered saline several times. An equal volume of a 0.5% suspension of chicken blood cells was added to each of the diluted allantoic fluids, followed by incubation at room temperature for 30 min. When hemagglutination is induced by viruses, a thin layer of agglutinated blood cells is formed on the bottom of a test tube.

Proliferation degree of influenza virus was evaluated by the hemagglutination assay.

As a result, VSF protein contained in both of the culture fluids and ascitic fluids was found to effectively inhibit proliferation of influenza virus, resulting in improvement of viability of the embryos. As shown in FIG. 4, when VSF protein was inoculated at the culture fluid state, the VSF protein was found to inhibit proliferation of influenza virus in a dose-dependant manner. In addition, as shown in FIG. 5, VSF protein contained in the ascitic fluids effectively suppressed proliferation of influenza virus, where the VSF protein was found not to negatively affect growth of the embryonated chicken eggs.

EXAMPLE 12: Evaluation for antiviral activity of VSF protein against HIV

10^4 SupT1 cells (human T cell line, Smith SD et al., Cancer Research, 44, 5657-5660, (1984)) were infected with about 10^3 $TCID_{50}$ of HIV-1 (NL strain). Simultaneously with the infection, the serially diluted ascitic fluid prepared in Example 2 was added to the cells. Then, the cells were cultured in RPMI 1640 medium (Gibco) containing 10% FCS at 37°C under 5% CO_2. Proliferation degree of HIV was evaluated by investigating formation of multinucleated giant cells called syncytia, which are formed 4 to 5 days after infection.

As shown in FIG. 6, when SupT1 cells were treated with the ascitic fluid containing VSF

protein, simultaneously with viral infection, VSF protein reduced syncytia formation in a dose-dependant manner.

In addition, the VSF protein contained in the ascitic fluid was found to have no negative effect on proliferation of the control cells.

EXAMPLE 13: Evaluation for inhibitory effect of VSF protein on induction of diabetes mellitus by EMCV

When SJL/J mice are infected with EMCV, they develop diabetes mellitus. In this test, it was investigated whether such induction of diabetes mellitus by EMCV is inhibited by the VSF protein. 9 week-old SJL/J mice (male) were divided into five groups, each of which comprised 6 mice according to administration and administration time of VSF protein. Group 1 was administered with only VSF protein, and Group 2 was administered with only EMC-D. Group 3 was administered with VSF protein simultaneously with infection with EMC-D. Group 4 was infected with EMC-D, and, after 4 hrs, administered with VSF protein. Group 5 was infected with EMC-D, and, after 24 hrs, administered with VSF protein. 10^4 pfu (0.5 ml) of EMC-D was intraperitoneally injected into mice. 0.5 ml of the culture fluid containing VSF protein was injected once into the tail vein of mice. From day 2 after infection with EMC-D, urine was collected from mice of each group every day to perform diagnosis of diabetes mellitus.

As shown in Table 5, below, and FIG. 7, no symptom of disease was found in Group 1 administered with only VSF protein, indicating that the VSF protein has no cytotoxic effect or side effects. In case of Group 2 administered with only EMC-D, all 6 mice showed severe diabetic symptoms on day 4 after viral infection, accompanied with reduction of body weight, and, moreover, two of the 6 mice died on day 10. In case of Group 3 administered with VSF protein simultaneously with viral infection, no diabetes mellitus was detected, and mice were in a healthy state. Also, in Group 4 administered with VSF protein 4 hrs after viral infection, no diabetes

mellitus was detected, and mice were in a healthy state. In case of Group 5, administered with VSF protein after 24 hrs of viral infection, diabetes mellitus was not developed, and reduction of body weight was not found.

These results demonstrate that the VSF protein has strong antiviral activity in vivo as well as in vitro.

In case of the conventional CAF, its antiviral activity was reported to rapidly decrease when being diluted, thereby not allowing in vivo experiments. Interferon is disadvantageous in terms of having remarkably reduced antiviral activity when being externally injected into the body, in comparison with when being naturally expressed in the body. In contrast, in this test, the VSF protein, only 0.5ml of culture fluid, showed an effect of perfectly inhibiting development of diabetes mellitus by EMCV, and was found not to be cytotoxic. These results indicate that the VSF protein has a potential to be developed to a novel drug.

Furthermore, such a perfect effect of inhibiting development of diabetes mellitus was also found even when the VSF protein was administered to mice 24 hrs before viral infection. This result indicates that the VSF protein is useful for prevention as well as treatment of viral infections.

TABLE 5

Development of diabetes mellitus in mice infected with EMC-D according to administration of VSF protein and its administration time

Group	Immediately before viral infection	Day 3 after viral infection	Day 4 after viral infection	Day 7 after viral infection
1	-	-	-	-
2	-	-	6/6	6/6
3	-	-	-	-
4	-	-	-	-

5	-	-	-	-

EXAMPLE 14: Evaluation for inhibitory effect of VSF protein on proliferation of HCMV

The culture fluid of the hybridoma 4D1B secreting VSF protein according to the present invention was evaluated for antiviral activity against HCMV (Human Cytomegalovirus). According to the same method as in Example 1, HCMV was treated with several dilutions of the culture fluid of the hybridoma 4D1B.

As a result, even when diluted 4 to 16 times, the culture fluid of the hybridoma 4D1B was found to perfectly inhibit proliferation of HCMV. This result supports the finding that the VSF protein has antiviral activity against a variety of viruses.

EXAMPLE 15: Determination of molecular weight of VSF protein by FPLC

The hybridoma 4D1B (accession number KCLRF-BP-00052) was cultured in 20% FCS-containing DMEM, and then SFM (serum-free medium) or PFM (protein-free medium). The medium was exchanged with a fresh medium at intervals of 2 days. The culture fluids were collected, and centrifuged at 1500×g to remove cell debris.

The supernatant was subjected to FPLC (Fast Flow Performance Liquid Chromatography) using a column filled with Superdex 200 HR 10/30 resin (Pharmacia Biotech, 17-1088-01). The supernatant was passed through the column at a flow rate of 0.5 ml/min. Active fractions containing VSF protein were collected by adding 0.05 M sodium phosphate buffer (pH 7.0) containing 0.15 M NaCl to the column.

As a result of SDS-PAGE, four major bands were, as shown in lanes 3 and 4 of FIG. 8, found in two about 163 kDa fractions. This band pattern was identical to SDS-PAGE results of active fractions obtained using three different columns, as shown in FIG. 1. This result indicates

that the VSF protein has a molecular weight of about 163 kDa.

EXAMPLE 16: Analysis of active fractions containing VSF protein by sucrose gradient centrifugation

The VSF protein according to the present invention was analyzed by sucrose gradient centrifugation to isolate subunits of the VSF multimer. A linear sucrose gradient (5-55%) was prepared in an ultracentrifuge tube using 5, 10, 20, 25, 42 and 55 % sucrose in Tris-HCl. The purified VSF protein was loaded onto the sucrose gradient, and ultracentrifuged at 160,000×g for 16 hrs using SW 41 rotor. After collecting fractions of 1 ml, SDS-PAGE was carried out.

As shown in FIG. 9, four major bands were found in the 17.11% sucrose fraction. Herein, a gel filtration marker was used as a standard size marker. The four major bands were identical to major bands of the active fractions obtained using three different columns, as shown in FIG. 1.

EXAMPLE 17: Evaluation for glycosylation of VSF protein

Using endoglycosidase H, it was investigated whether the VSF protein is glycosylated.

Endoglycosidase H cleaves bonds between oligosaccharides or polysaccharides and protein by hydrolysis.

The hybridoma 4D1B (accession number KCLRF-BP-00052) prepared in Example 1 was cultured in SFM (serum-free medium, Gibco) at 37°C under 5% CO_2. Thereafter, endoglycosidase H was added to the culture fluid, followed by incubation at 37°C for 1, 12 and 24 hrs.

As shown in FIG. 10, when the VSF protein treated with endoglycosidase was separated on a SDS-polyacrylamide gel, there was no band shift. This result indicates that the VSF protein

is not glycosylated.

EXAMPLE 18: Evaluation for sensitivity of VSF protein to proteinases

The VSF protein was evaluated for sensitivity to some proteinases. The hybridoma 4D1B (accession number KCLRF-BP-00052) prepared in Example 1 was cultured in SFM (Gibco) at 37°C under 5% CO_2. Trypsin, cathepsin, papain and pepsin were separately added to the culture fluid. Trypsin used in this test had a specific activity of 10,000 BAEE unit/mg protein. 250 μg of trypsin was added to 20 μg of VSF protein, followed by incubation at 37°C for 1, 6 and 24 hrs. 28 units of cathepsin, which has a specific activity of 33 units/mg protein, was added to 20 μg of VSF protein, followed by incubation at 37°C for 24, 48 and 72 hrs. Papain was added to 20 μg of VSF protein at various amounts of 100 ng, 1 μg and 5 μg, followed by incubation at 37°C for 30 min. Pepsin was added to 20 μg of VSF protein at various amounts of 100 ng, 1 μg and 10 μg, followed by incubation at 37°C for 30 min. After completion of reaction, the culture fluid was analyzed by SDS-PAGE and Western blotting.

As shown in FIGs. 11A to 11D, versus trypsin cleavage, the mouse IgG control showed weak resistance in both light and heavy chains, while the H subunit of the VSF protein was very sensitive, and the L1, L2 and L3 subunits were virtually unaffected by the trypsin treatment. Also, the VSF protein was not cleaved by cathepsin. In case of being treated with papain, the H subunit of the VSF protein and the heavy chain of mouse IgG showed different reactivities to anti-mouse IgG gamma heavy chain. In addition, when compared to the control mouse IgG heavy chain, the H subunit of the VSF protein showed similar susceptibility to pepsin cleavage, but displayed different reactivity to anti-mouse IgG Fab-specific antibody.

These results indicate that the H subunit of the VSF protein is similar to mouse IgG, but the L1, L2 and L3 subunits are very distinct from mouse IgG.

EXAMPLE 19: Evaluation for heat stability of VSF protein

This test was performed to determine a suitable temperature range for long-term storage and use of the VSF protein. The hybridoma 4D1B (accession number KCLRF-BP-00052) prepared in Example 1 was cultured in SFM (Gibco) at 37°C under 5% CO_2. The resulting culture fluid was incubated at 37°C, 56°C and 65°C. Thereafter, antiviral activity was analyzed by the staining method using crystal violet as described in Example 1.

As shown in FIGs. 12A to 12C, the VSF protein contained in the culture fluid was found to be stable for 3 days at 37°C, and about 40 min at 56°C. However, at 65°C, the VSF protein lost over 50% of its antiviral activity within 5 min.

EXAMPLE 20: Evaluation for pH stability of VSF protein

This test was performed to determine a suitable pH range for long-term storage and use of the VSF protein. The hybridoma 4D1B (accession number KCLRF-BP-00052) prepared in Example 1 was cultured in SFM (Gibco) at 37°C under 5% CO_2. The resulting culture medium was incubated at a broad range of pH 2.0 to 12.5 for 20 min, 1 hr and 3 hrs. Thereafter, antiviral activity was analyzed by the staining method using crystal violet as described in Example 1. As a result, the VSF protein was found to be stable in a range of pH 7.0 to 8.0.

EXAMPLE 21: Analysis of primary structure of VSF protein

The VSF protein was analyzed for primary structure by MALDI-TOF, N-terminal sequencing and internal sequencing. MALDI-TOF was performed by Genomine, and N-terminal sequencing and Internal sequencing were performed by Korea Basic Science Institute (KBSI), Seoul, Korea. In addition, based on the similarity to mouse immunoglobulin, referring to its

known nucleic acid sequences, PCR was carried out to mRNA which was isolated from the hybridoma 4D1B, and, from the resulting amplified products, amino acid sequences were obtained and compared to the amino acid sequences got from MALDI-TOF, N-terminal sequencing and internal sequencing, resulting in finding of genes encoding the H and L3 polypeptides.

As a result, the H polypeptide of the VSF protein was found to have a DNA sequence and an amino acid sequence designated SEQ ID NO: 1 and SEQ ID NO: 2, respectively. The L3 polypeptide was found to have a DNA sequence of SEQ ID NO: 3 and an amino acid sequence of SEQ ID NO: 4. With respect to a result of BLAST search with GeneBank DataBase, as well as molecular weight, amino acid sequence and reactivity to protein A and anti-mouse IgG, the C-termini of the H and L3 polypeptides appear to be identical or very similar to mouse Ig γ3 chain and mouse Ig κ chain, respectively. In addition, the C-termini of the L1 and L2 polypeptide were found to have some amino acid homology with mouse Ig κ chain.

EXAMPLE 22: Analysis of active fractions containing VSF protein by Western blotting

The active fractions containing VSF protein prepared in Example 6 were run on a SDS-PAGE gel and analyzed by Western blotting using anti-mouse IgG(H+L) antibody, anti-mouse gamma heavy chain-specific antibody, anti-mouse Fab-specific antibody and anti-mouse kappa light chain-specific antibody.

As a result, when performing Western blotting using anti-mouse IgG(H+L) antibody and anti-mouse Fab-specific antibody, all of four major bands as shown in FIG. 1 were detected. In case of Western blotting with anti-mouse gamma chain-specific antibody, only one band (H) of the four major bands was detected around 55 kDa. In case of Western blotting with anti-mouse kappa light chain-specific antibody, three bands (L1, L2 and L3) were detected around 30 kDa and 25 kDa.

EXAMPLE 23: Evaluation for inhibitory activity of VSF protein against VSV

The culture fluid of the hybridoma 4D1B secreting VSF protein was evaluated for antiviral activity against VSV (Vesicular Stomatitis Virus). According to the same method as in Example 1, VSV was treated with various dilutions of the culture fluid of the hybridoma 4D1B.

As a result, the undiluted culture fluid of the hybridoma 4D1B was found to perfectly suppress proliferation of 100 pfu of VSV. This result supports that the VSF protein has antiviral activity against a variety of viruses.

EXAMPLE 24: Comparison of reactivity of VSF protein and mouse IgG to anti-mouse IgG(H+L) antibody by ELISA

The VSF protein according to the present invention was evaluated for reactivity to anti-mouse IgG, in which mouse IgG was used as a positive control. The culture fluid of the hybridoma 4D1B secreting VSF protein was put into a culture vessel to which anti-mouse IgG was attached in advance, and peroxidase-conjugated anti-mouse IgG was added to the culture vessel. After reaction, optical density was measured at 490 nm. The resulting O.D value was found to be some lower (FIG. 13) than the positive control mouse IgG. This result indicates that the VSF protein contains some components similar to mouse IgG.